

Spinosad Induced Cytogenotoxic Effects on the Mosquito Fish, *Poecilia reticulata*

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Abstract

Background: Spinosad is an insect control product developed initially for agricultural pests. In recent times, the bio-rational compound has gained popularity in the area of mosquito larval control. The cytogenotoxic potential of the naturally derived compound was investigated on the mosquito fish, *Poecilia reticulata* to assess its compatibility as a potential larvicide for integrated mosquito larviciding.

Methods: Blood samples were collected from the gill epithelial cells of *Poecilia reticulata* exposed in vivo to three concentrations of Spindor dust (60 µgL⁻¹, 123 µgL⁻¹, 361 µgL⁻¹). The frequencies of micronucleus and other nuclear abnormal cells as well as, normochromatic cells from treated and untreated media were evaluated after sacrificing the fish at sampling times of 1, 3, 7, 14, 21 and 28 days.

Results: The induction of micronucleus, nuclear abnormal and normochromatic cells were highly significant (P<0.01; P<0.001). Polychromatic erythrocytes were more sensitive than binucleated cells and this occurred with increasing concentration of the larvicide (P<0.01). The ratio of polychromatic cell to normochromatic cells increased significantly by concentration and time of exposure when compared with control (P<0.05).

Conclusion: The naturally derived Spinosad inhibited mitotic division in *Poecilia reticulata* therefore it was not compatible with the fish species for integrated mosquito larviciding at the exposed concentrations.

Keywords: Cytogenotoxicity; Spinosad; *Poecilia reticulata*; Micronucleus

Introduction

The mosquito fish *Poecilia reticulata*: (Poeciliidae) are conspicuous members of many freshwater ecosystem in Nigeria such as drainages, standing water and streams which make up the usual habitat for mosquito larvae. Consequently, these bodies of water are targets of larvicidal activities during mosquito larval control practices. Owing to the deleterious effects of synthetic insecticides particularly on non-target species, there appears to be a growing interest in the use of alternative insecticides that are more environmentally safe.

Spinosad is an insect control product derived from the fermentation of a soil bacterium *Saccharopolyspora spinosa*. It represents a new generation of bio-rational insecticides developed initially for the control of agricultural pests with a reduced spectrum of toxicity compared to the synthetic insecticides [1]. The low toxicity of spinosad to non-target organisms, particularly mammals, have earned it the name: 'Reduced Risk Material' by the US Environmental Protection Agency [2], and this has contributed to its continual use by Integrated Pest Management (IPM) Practitioners since the 1980s [1]. The action of spinosad on the insect is a unique one, acting on the post synaptic nicotinic acetylcholine and Gama Aminobutyric Acid (GABA) receptors [3,4]. Spinosad was not affected by the existing resistance mechanism to conventional insecticides and is highly toxic to mosquito species [5-8]. These properties of spinosad especially its low mammalian toxicity and larvicidal potentials, have contributed to the strong and growing recommendations of the compound as a replacement for synthetic organophosphates in domestic and urban mosquito larval control [6,9]. The need for an accurate assessment of the environmental impact of insecticides on non-target organisms is an issue of international concern especially now that spinosad is presently under review for large scale mosquito control usage [9]. Thus far, most toxicity tests done with spinosad has focused on acute toxicity bioassays [8,7].

Poecilia reticulata (guppy) are excellent model for genetic monitoring of toxic chemicals in aquatic environment [10,11]. They can accumulate toxic substances and respond to low concentrations of mutagens [12]. Hence they have become a very important sentinel organism to assess the cytogenotoxicity of larvicides in aquatic population. Guppies adapt very well in mosquito larval habitat and play a number of roles in the regulation of mosquito larvae population [13] hence; they could be integrated with larvicides in the control of mosquito species. This study is aimed at investigating the compatibility of spinosad as a potential larvicide for integrated mosquito larval control by assessing some cytogenotoxic parameters on a biological control agent, the guppy fish. The data generated will assist in the establishment of a dosage solution for integrated mosquito larval control practice involving the use of fish species as a support control agent.

Materials and Method

Collection of guppy

Guppies were collected between 6.30 am and 9.00 am from drainage at Christian Missionary Society (CMS) Grammar School, Bariga, and Lagos using a fish net of mesh size 1.5 mm in diameter.

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They were immediately transported to the laboratory in a transparent plastic bucket containing some drainage water.

Rearing of guppy

Guppies were gently released into a holding tank of capacity 200 L containing dechlorinated tap water at pH 7. The fish were reared under laboratory condition of 28°C ± 0.8°C, 72% ± 2% RH and, 12 h light and 12 h dark regime. The tank was drained then washed and refilled with fresh dechlorinated tap water twice weekly to prevent the accumulation of fish metabolic wastes. After 8 days of acclimatization period, selected brood stocks were transferred into 5 L plastic containers to obtain offspring. After 3–4 weeks period of completion of a cycle of reproduction, 2 day old juveniles were separated from adults and introduced into 2 L well-aerated dechlorinated tap water where they were allowed to mature into adult sizes of mean length 3.5 ± 0.2 cm.

Physico-chemistry of test media

The physico-chemical characteristics (pH, dissolved oxygen, conductivity, temperature and salinity) of the test media and dechlorinated tap water (control) were analyzed with a pH meter (©Mettler Toledo AG), DO meter (©Mettler Toledo AG), Conductivity meter (©Mettler Toledo AG), Stem Glass Thermometer (Uniscopex), and Master Refractrometer (Atago, Japan), respectively.

Micronucleus assay

Low concentrations of spinosad that were within the range that killed 40%, 75% and 98% of *Culex* mosquito larvae were computed and used for the study because at preliminary toxicity study by Anogwih [14], the 24 hLC₅₀ value for the fish was indeterminate. *Poecilia reticulata* were not fed 24 h before testing and the static renewal test technique was adopted where the test media were renewed at the same concentration once every 48 h [15]. Sixty three fish of mean length 3.5 ± 0.2 cm was randomly selected and divided into 3 groups (21 fish/group). x Each group was exposed to the established concentrations of spinosad (60 µg/L⁻¹, 123 µg/L⁻¹ and 361 µg/L⁻¹) for 28 days. Fish were checked every 24 h and dead fish were removed immediately. From each replicate and control, 3 fish were randomly sampled for structural aberrations including micronucleus at days 1, 3, 7, 14, 21 and 28 respectively using the conventional Giemsa protocol as described by Campana et al. [16]. Gill cells were collected from the gill arches of each fish and smeared on three clean slides. The cells were then fixed in absolute ethanol for 20 minutes and air dried. After 24 h, each slide was stained in May-Grünwald for 6 minutes and in 15% Giemsa solution for 10 minutes. Stained slides were rinsed thoroughly with distilled water and left to air dry. Slides were randomly selected and coded. From each slide, the frequencies of Micronucleated cells, Binucleated cells, Poly chromatic or immature cells and Normochromatic or mature cells were determined for 3000 cells at 63x/1.4 oil immersion (Zeiss Axio Imager Microscope). PCEs were identified as young cells without visible cytoplasmic boundary [17], BN as cells with two nuclei of relatively equal size bounded as in mature cells or unbounded as in immature cells [12,18], and Normo-chromatic erythrocytes as normal cells with distinct cytoplasmic boundary [19]. At least one out of the following criteria was used to identify micronucleus (a) MN must be smaller than one-third of the main nuclei; (b) MN must be clearly separated from the main nuclei; (c) MN must be on the same plane of focus and have the same colour of stain as the main nucleus [18].

Statistical analysis

The Student paired sample T-test from SPSS Version 15.0 for Windows (SPSS Inc. Chicago, IL, USA) was used to analyze the

significant differences in the frequency of micronucleus and nuclear abnormal cells in treated and control media. Two-Way Anova from Graph Pad Prism Version 5 for windows (GraphPad software, Inc. CA, USA) was used to find the significant differences between PCE/NCE from control and treated after calculating the percentage (%) ratio according to Pacheco and Santos [20], as follows:

$$\text{PCE frequency (\%)} = \frac{\text{No PCEs}}{\text{No. PCEs+NCEs}} \times 100$$

Results

Physico-chemistry of test media

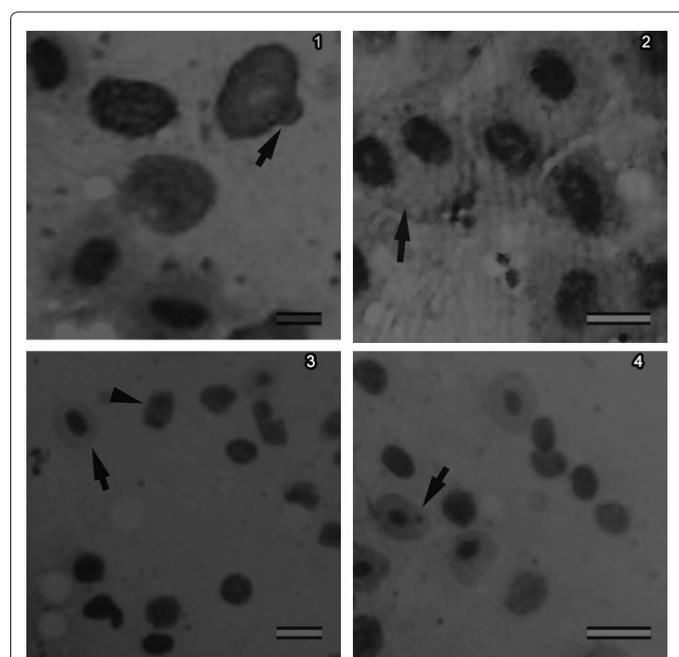
During the experiment, the physico-chemical characteristic of the test media remained fairly stable and did not quite differ from the control (Table 1).

Representative photomicrograph of guppy gill cells

The cells of guppy have centrally placed round nuclei and a sizeable cytoplasm (Plates 1-4). The size as well as, the location of the micronucleus within the cytoplasm varied from cell to cell and the shape was oval in almost all the cells. The nucleus has a well-defined

Parameters	Control	Spinosad
pH	6.80	6.75
Salinity	0.00‰	2.5‰
Conductivity	0.09 mgL ⁻¹	0.09 mgL ⁻¹
Dissolved Oxygen (DO)	4.90 mgL ⁻¹	5.00 mgL ⁻¹
Temperature	23.0°C	23.1°C

Table 1: Mean physicochemistry of test media.



Plates 1-4: Guppy gill cell stained with 15% giemsa and observed under Zeiss Axio Imager microscope at 63x/1.4 oil immersion.

- 1: Micronucleated polychromatic cell (arrow)
- 2: Binucleated normo-chromatic cell (arrow)
- 3: Binucleated polychromatic cell (arrowhead); Normal cell with distinct cytoplasm (arrow)
- 4: Micronucleated Normal cell (arrow). Scale bars = 10µm each

boundary distinctly larger than the MN fragments which facilitated its ease of identification. Single MN was generally seen in most affected cells but there were incidences of MN occurring with PCE cells (Plate 1).

Concentration dependent cell types

The frequency of MN in the treated experiment increased with decreasing concentration of spinosad (Table 2). There was no induction of MN in the control experiment and the increase in MN between the control and treated cells was highly significant at $P < 0.01$; $P < 0.001$ (Table 2). The frequency of nuclear abnormal cells (NA) other than MN in the fish varied insignificantly ($P > 0.05$) from control. Of the two types of nuclear abnormal cells analyzed, PCE was faster to manifest than BN (Table 3). On Table 4, the repression of mature cells in the treatment was not significantly different from control ($P > 0.05$). The percentage (%) ratio of PCE to NCE increased in the exposed gill cells of *P. reticulata* with concentration and time of exposure when compared to the control (Figure 1).

Time dependent cell types

There were no clear concentration–time dependent frequencies

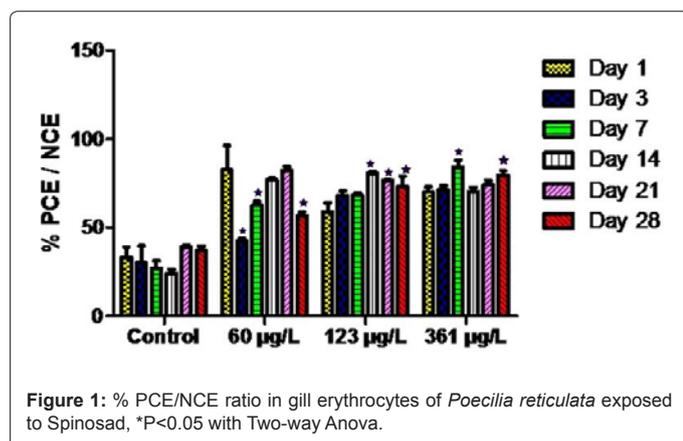


Figure 1: % PCE/NCE ratio in gill erythrocytes of *Poecilia reticulata* exposed to Spinosad, * $P < 0.05$ with Two-way Anova.

Treatment	Conc ($\mu\text{g/L}^{-1}$)	Tot Av. Cells analyzed (N × 3000)	MN (Mean % ± SE)
Control	0	54,000	0 ± 0.000
Spinosad	60	54,000	0.018 ± 0.003 ^{ooo}
	123	54,000	0.002 ± 0.004 ^{oo}
	361	54,000	0.002 ± 0.001 ^{ooo}

N=Sampling time × Nos of replicates (3)

^{ooo} $P < 0.001$; ^{oo} $P < 0.01$; ^o $P < 0.05$ with Student T-test

Table 2: Frequency of Micronuclei cells in *Poecilia reticulata*.

Treatment	Conc ($\mu\text{g/L}^{-1}$)	Tot. Av. Cells (N × 3000)	PCE	BN	Tot. NA cells (PCE+BN)	NCE
Control	0	54,000	1.054 ± 0.193	1.187 ± 0.105 ^{ns}	2.323 ± 0.239	2.122
Spinosad	60	54,000	1.631 ± 0.292 ^o	1.053 ± 0.121 ^{ns}	2.684 ± 0.296 ^{ns}	0.666 ± 0.170 ^{ooo}
	123	54,000	1.844 ± 0.186 ^o	0.894 ± 0.122 ^{ns}	2.738 ± 0.264 ^{ns}	0.634 ± 0.061 ^{ooo}
	361	54,000	2.079 ± 0.305 ^{oo}	0.913 ± 0.089 ^{ns}	2.992 ± 0.245 ^{ns}	0.629 ± 0.151 ^{ooo}

N=Sampling time × Nos of replicates (3)

BN (binucleated cell); PCE (polychromatic cells); NA (Nuclear abnormal cells); MN (micronucleus)

^o $P < 0.05$; ^{oo} $P < 0.01$; ^{ooo} $P < 0.001$; ^{ns} $P > 0.05$ with Student T-test

Table 3: Frequencies of Nuclear Abnormal Cells in *Poecilia reticulata* (Mean% ± SE).

Treatments	Time of Exposure (Days)					
	1	3	7	14	21	28
Control	1.5 ± 2.121	1.356 ± 1.918	1.150 ± 1.626	1.255 ± 1.775	0.557 ± 0.787	0.548 ± 0.775
Spinosad	0.718 ± 1.015	1.505 ± 2.128	0.718 ± 1.015	0.600 ± 0.846	0.580 ± 0.792	0.863 ± 1.220

$P > 0.05$ with Student T-test

Table 4: Repression of normochromatic cells in *Poecilia reticulata* (Mean% ± SD).

for MN, NA and NCE cells respectively but some variations in the pattern of induction of these cell types are shown in Figures 2a-2c respectively. With respect to MN, and under the highest concentration of the larvicide, differences in the elevated response peaked at Day 3 while at its lowest concentration; peak effect was attained at Day 14 (Figure 2a). Considering the NA cells, peak induction occurred at Day 1 under the highest concentration of Spinosad and decreased thereafter. However, at its lowest concentration, there was an initial increase in the induction of NA cells that peaked at Day 14 (Figure 2b). At the highest concentration of Spinosad, mature cells (NCE) were greatly repressed at Day 7 while at its lowest concentration; the repressive effect was gradual but peaked at Day 21 (Figure 2c).

Discussion

In this study, an in vivo genotoxic and cytotoxic effects of spinosad on the gill cells of guppy were investigated by using the Micronucleus (MN), Nuclear abnormality (NA) and the ratio of Polychromatic erythrocytes to Normo-Chromatic Erythrocytes (% PCE/NCE) tests respectively. MN test is considered as the most suitable and effective method to use when evaluating the genotoxic (functional) effects of xenobiotics on fish species because of its simplicity and ease of scoring [21]. Micronuclei are small fragments of intra-cytoplasmic chromatin which arise from chromosome breaks or whole chromosomes after the action of clastogenic substances or spindle-poisons that do not migrate during anaphase [16,12].

Spinosad induced a significant MN in the fish gill cells which implies that the bio-rational compound had the capability to inhibit cell division and thereby could affect growth in the exposed guppy fish. Surprisingly, the incidence of MN in guppy exposed to spinosad increased with decreasing concentration of the larvicide. The reason for this is not clear but similar results by some researchers who worked on different compounds have been obtained [22,16].

The total absence of MN in the control (untreated dechlorinated tap water) clearly indicated that the MN had certainly been induced by the test larvicide. This result was unexpected since guppies are known to inhabit gutters and open drains, thus, are usually prone to contamination. It is possible that several mitotic divisions of the initially induced MN from drain pollutants were eventually lost as a result of the long period of laboratory rearing of the fish prior to bioassay.

Some variations existed in the time of induction of MN and NA cells in the fish. The elevated response in the frequencies of MN that were found at the highest concentration of the larvicide was most pronounced on day 3. At its lowest concentration however, damage to

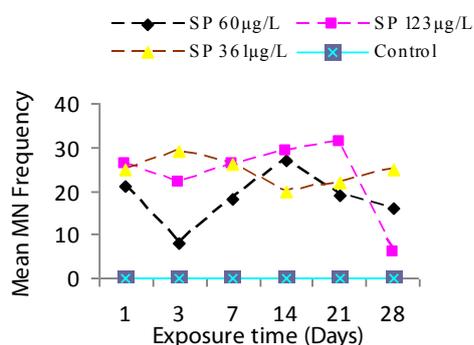


Figure 2a: Pattern of Micronucleus induction with Spinosad.

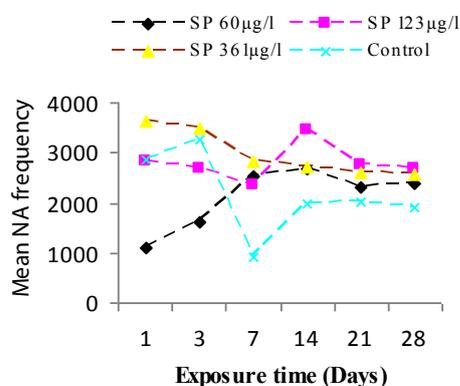


Figure 2b: Pattern of Nuclear Abnormal cell induction with Spinosad.

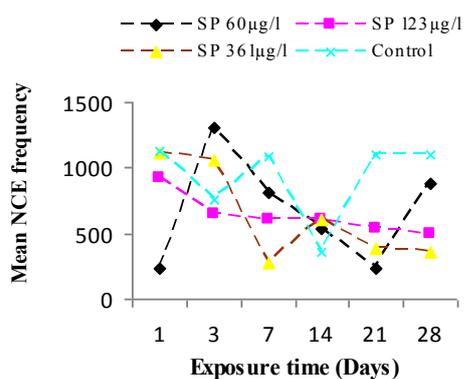


Figure 2c: Pattern of Normo-chromatic cell induction with Spinosad.

the fish cells peaked on day 14. The explanation for this could be that the inhibitory effect on mitotic division by the compound was more lasting at the lowest concentration of spinosad hindering the passage of the affected cells into the tissue erythrocyte at this concentration compared to the highest concentration where growth inhibition was short-lived. Earlier researchers have demonstrated various time dependent MN responses. Cavas and Ergene-Gozukara [18] showed a significant increase in MN only at the highest concentrations and longest exposure duration on treating *Oreochromis niloticus* with petroleum and chromium processing effluents. A gradual decrease in

the frequency of MN with an increase in exposure periods was also obtained by Das and Nanda [22] who worked with paper mill effluents on a fish species.

The cumulative analysis of the NAs (PCE & BN) induced by the larvicides showed an insignificant increase from the control indicating that spinosad was not cytotoxic to the fish. The elevated response in the nuclear abnormal cells was most pronounced on day 1 compared to the MN induction that peaked on day 3 at the highest concentration of spinosad. The rapid response of NA cells compared to MN induction is indicative that NA cells were very prompt to detect negative influences from spinosad making it a better cytological biomarker than MN. It is important to note that this rapid response by NA was highly contributed by PCE cells. Carlin and Dragonir [23]; Archipchuk and Garanko [24] have also reported similar result upon the exposure of fish and rat cells to different compounds such as benzo(a)pyrene, N-acetylaminofluorene, cadmium and copper.

The pattern of induction of NAs in organisms depends on the genetic system or assay used [16]. Cavas and Ergene-Gozukara [18] reported an insignificant increase in NAs in *Oreochromis niloticus* exposed to chromium effluent. In a similar but different study, they observed a significant repression of all analyzed nucleolar parameters in fin cells of a fish species exposed to lambda-cyhalothrin [12]. Various assumptions have been propounded on the mechanism underlying the formation of NAs: they may result from problems segregating tangled and attached chromosomes [12]; gene amplification via the breakage-fusion-bridge cycle could cause NAs like lobed nuclei and blebbed nuclei during the elimination of amplified DNA from the nucleus. Many authors have maintained that NAs are induced in response to exposure to genotoxic agents [25-27].

The repression of normal cells was observed in both control and treatment group although; the result was insignificantly different from each other. However, the decrease in response was more in the treatment group than in the control indicating that the larvicides were definitely responsible for the repressive activity found in the treated cells. This was further supported by the observed peak periods of NCE repression that coincided with the periods of change of the bioassay. The repressive response observed in the control may be associated with external stress factors including fish handling and the laboratory conditions in which the fish were subjected to.

As a possible parameter of mutagen-induced cytotoxicity, the ratio of PCE to NCE in the fish gill cells was assessed and this showed a significant increase with relation to concentration and time. This infers that at the tested concentrations, the cytotoxic effect of the larvicide on guppy fish was not mutagenic. Decreases in the proportion of immature erythrocytes (PCE) to mature or normochromatic erythrocytes (NCE) are considered as an indicator of mutagen-induced cytotoxicity [28]. Therefore, PCE/NCE ratio is a key component of cytotoxicity assessment routinely included in micronucleus tests with mammalian test organisms [29,30]. However, there is a dearth of information on the combination of PCE/NCE ratio with micronucleus in fish toxicity tests [19]. Pacheco and Santos, [20] reported that PCE frequencies in peripheral blood of *Anguilla anguilla* decreased while erythrocyte micronucleus frequencies increased as a result of Benzo[a]pyrene, dehydroabietic acid and bleached kraft paper mill effluent treatments. This study has demonstrated the combination of PCE/NCE ratio with micronucleus and nuclear abnormal cells in *P. reticulata* gill cells exposed to spinosad larvicide contributing immensely to the dearth of information in this area of research.

Conclusion

The present study has shown that the naturally derived spinosad was genotoxic by inhibiting mitotic division in *P. reticulata* therefore is not likely to be compatible for use in integrated mosquito larviciding at the tested concentration. Spinosad merits a further and detailed toxicity/safety evaluation at reduced concentrations before its recommendation for use in field mosquito larval control whether in a single or integrated approach.

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